Arbuscular Mycorrhizas and Dark Septate Fungi in *Lycopodium paniculatum* (Lycopodiaceae) and *Equisetum bogotense* (Equisetaceae) in a Valdivian Temperate Forest of Patagonia, Argentina

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Abstract.—Arbuscular mycorrhizas (AM) are one of the most widespread and common type of symbiotic associations. *Lycopodium paniculatum* and *Equisetum bogotense* are two important species of seedless vascular plants in a Valdivian temperate forest of Patagonia, Argentina. The mycorrhizal status of these species is completely unknown, as it is for most lycophytes and monilophytes in Argentina, where information on symbiotic interactions in these plants is scarce. In this study, typical AM structures were observed in sporophytes of *L. paniculatum* and *E. bogotense*. The percentage of root length colonized by AM fungi ranged from 0 to 50% in the first species and from 0 to 22.5% in *E. bogotense*. Both species were facultative mycorrhizal and it was observed that the habitat and substrate seem to play an important role in determining the colonization intensity. The morphological AM colonization pattern was considered an *Intermediate*-type. Dark septate fungi, characterized by septate hyphae and microsclerotia, were also present within the roots of *L. paniculatum* and *E. bogotense*.

Key Words.—arbuscular mycorrhiza, morphological type, *Intermediate*-type, dark septate fungi, *Lycopodium*, *Equisetum*, Valdivian temperate forest

Mycorrhizas are symbiotic associations, usually mutualistic, that improve plant fitness and influence plant biodiversity and productivity in natural ecosystems (Brundrett, 1991; Read, 1991; Smith and Read, 1997). Arbuscular mycorrhizas (AM), as the most widespread type of mycorrhizas, form symbiotic interactions with the roots of ~80% of all terrestrial plant species (van der Heijden *et al.*, 1998; Read, 1999). There are two major morphological types of AM: *Arum* and *Paris*-type. In the *Arum*-type the fungi form extensive intercellular hyphae in the root cortex and then develop short lateral branches into cortical cells forming arbuscules as terminal structures on trunk hyphae.

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In contrast, in the *Paris*-type intercellular hyphae are lacking and the fungi spread directly from cell to cell forming extensive coils frequently developing intercalary arbuscules (structures known as "arbusculate-coils") (Smith and Smith, 1997).

Seedless vascular plants (including extant lycophytes and monilophytes (Kranz and Huss, 1996; Pryer *et al.*, 2001, 2004; Smith *et al.*, 2006)) are of ancient origin and occupy a very important position in the origin and evolution of vascular plants (Remy *et al.*, 1994). Arbuscular mycorrhizas have been found in some fossil seedless vascular plants, and in spite of having persisted for 400 million years (MY) (Pirozynski and Malloch, 1975; Malloch, 1987; Hass *et al.*, 1994; Remy *et al.*, 1994), the AM status of extant lycophytes and monilophytes is poorly understood (Dhillion, 1993; Zhao, 2000; Zhang *et al.*, 2004).

Some authors (Boullard, 1957; Hepden, 1960; Freeberg, 1962; Cooper, 1976; Iqbal *et al.*, 1981; Berch and Kendrick, 1982; Koske *et al.*, 1985; Harley and Harley, 1987; Duckett and Ligrone, 1992; Gemma *et al.*, 1992; Schmid and Oberwinkler, 1993, 1994; Swatzell *et al.*, 1996; Treu *et al.*, 1996; Turnau *et al.*, 2005; Muthukumar and Udaiyan, 2000; Zhao, 2000 and Zhang *et al.*, 2004; Winther and Friedman, 2007) have studied the occurrence of AM interactions in sporophytes and gametophytes of different seedless vascular plants, including several *Lycopodium* and *Equisetum* species. According to Read *et al.* (2000) and Winther and Friedman (2007), *Lycopodium* gamethophytes appear to be obligatory mycotrophic, but there is little consensus regarding the AM associations in the sporophytes. On the other hand, Read *et al.* (2000) point out that those surveys concerning the mycorrhizal status of different *Equisetum* species have shown that the gametophytic generation seems to be non-mycotrophic and that the sporophytes could be non-mycorrhizal as well as capable of forming AM. From this analysis, it becomes evident that a significant discrepancy exists in our knowledge of the occurrence of fungal symbioses in *Lycopodium* and *Equisetum* species.

In addition to AM, another type of root colonizing fungi, called dark septate fungi (DSF), has been also reported within the roots of different seedless vascular plants (Cooper, 1976; Berch and Kendrick, 1982; Dhillion, 1993; Jumpponen and Trappe, 1998). Dark septate fungi are defined by Jumpponen (2001) as conidial or sterile fungi that colonize living plant roots without causing any apparent negative effects. The ecology, taxonomic affinities and host range of these DSF are largely unknown, as is their influence on the host and plant communities (Peterson *et al.*, 2004). Including DSF in mycorrhizal studies would yield valuable information about the importance and frequency of these root colonizers (Jumpponen and Trappe, 1998).

Seedless vascular plants are abundant among the vegetation of the Valdivian temperate forests of South America, which are characterized by their levels of endemism and the antiquity of their ecological interactions. This ecoregion is located in the Southern area of Argentina and Chile, running from 35° to 48° S latitude, between the Eastern slope of the Andes and the Pacific Ocean (Cabrera, 1976; Armesto *et al.*, 1995). This study was carried out in Puerto

Blest, which is situated in the Valdivian temperate forest region of Argentina. Two important seedless vascular plants species present in this region are *Lycopodium paniculatum* Desv. ex Poir. and *Equisetum bogotense* HBK. The first species is endemic to the temperate forest of Argentina and Chile (between 38° and 48° S latitude), while *E. bogotense* has a wider geographical distribution and is restricted to moist habitats (close to streams, rivers and lake coasts) of the Andean mountain region (Correa, 1998). Currently, the mycorrhizal status of these two species it is completely unknown.

The aim of the present study was to analyze the occurrence and abundance of mycorrhizal symbionts in sporophytes of the endemic *L. paniculatum* and the more widely distributed *E. bogotense* in Puerto Blest, a Valdivian temperate forest of Patagonia. *Lycopodium paniculatum* and *E. bogotense* are the only species of each genus growing in this Valdivian temperate forest, and they are relatively rare compared to other non-seed plant species. A common feature between these species that distinguish them from the rest of the seedless vascular plants of Puerto Blest is the presence of microphylls (Brion *et al.*, 1988; Gifford and Foster, 1989; Sitte *et al.*, 2004). *Lycopodium paniculatum* and *E. bogotense* specimens were sampled from the forest dominated by *Nothofagus dombeyi* (Mirb.) Oerst. and a waterlogged peat bog because these are the most important vegetation units of this Valdivian temperate forest.

MATERIALS AND METHODS

Study Area.—This study was carried out in Puerto Blest (41° 02' S; 71° 49' W), within the Nahuel Huapi National Park in Río Negro Province, Patagonia, Argentina. Puerto Blest is part of the Valdivian temperate forest region of Argentina. It is one of the rainiest places in the country and its average annual rainfall is 3000 mm. The annual average temperature is 9° C (Dimitri, 1972; Brion *et al.*, 1988). In this hydrophilic forest, *N. dombeyi* is the dominant tree species. However, in some areas it forms mixed forests with *Fitzroya cupressoides* (Molina) I. M. Johnst., *Saxegothaea conspicua* Lindl., *Dasyphyllum diacanthoides* (Less.) Cabrera and *Laureliopsis philippiana* (Looser) Schodde. Species such as *Lomatia ferruginea* (Cav.) R. Br. and *L. hirsuta* (Lam.) Diels *ex* J. F. Macbr., *Desfontainea spinosa* Ruiz and Pav., *Azara lanceolata* Hook. *f.*, *Berberis darwinii* Hook., *Fuchsia magellanica* Lam. and *Chusquea culeou* Desv. are very common in the shrub-layer. Within Puerto Blest forest there is a waterlogged peat bog, which corresponds to a water-retaining *Sphagnum magellanicum* Brid. prairie where *F. cupressoides* and *Pilgerodendron uviferum* (D. Don) Florin are the dominant tree species (Roig, 1998).

Sampling procedures.—Sporophytes of *L. paniculatum* and *E. bogotense* were collected in autumn of 2006 in Puerto Blest. The forest dominated by *N. dombeyi* and the waterlogged peat bog were chosen as sampling environments because they are the most representative vegetation units of this Valdivian temperate forest.

Specimens were collected from terrestrial and epiphytic habitats such as: soil in the forest, soil at the edge of the road, soil of the coast of the lake and trunk surfaces. Due to the presence of a road, soil is usually removed and there were two different conditions on its edges: stony and sandy soil; it was observed that the last one usually has higher soil moisture than the former. Sporophytes were sampled by random walk and were carefully removed from the substrate with a shovel. Each specimen was stored in a labelled plastic bag and refrigerated at 4° C until further procedures. Afterwards, roots from all samples were first excised from the rest of the plant, and then carefully rinsed with tap water, cleaned under a stereoscopic microscope (Olympus SZ 30) and fixed in 70% formalin-acetic acid-alcohol (FAA).

Clearing and staining.—All samples were stained using a modified Phillips and Hayman (1970) method. Roots were first cleared with 10% (w/v) KOH at 98° C for 1 h. After clearing, *L. paniculatum* roots were bleached with a 5% (v/v) H_2O_2 (30V) and 0.5% NH_3 solution for 10 minutes. *Equisetum bogotense* roots were darker, so they were bleached in this solution for 40 minutes. All samples were acidified with 1% v/v HCl for 5 minutes and stained with Trypan Blue in acidic glycerol (31% v/v glycerol, 31% v/v lactic acid and 0.05% w/v Trypan Blue) by heating them at 98° C for 15 minutes. The stained roots were stored in acidic glycerol.

Analyses of the samples.—For each specimen, ten stained root pieces approximately 1 cm long were mounted on a slide in Glycerol and were examined with a light microscope (Olympus BX40). A total of three replicates of each individual were made. Typical structures that indicated the presence of mycorrhizas or other root associated fungi, such as DSF, were documented as brightfield images, which were captured with a digital camera (Sony ExwaveHAD) and Image-Pro Plus 4.1.0.0. analysis software for Windows.

The mycorrhizal type present in each sample was designated according to Harley and Smith (1983) classification. If one of the species was found to be mycorrhizal in one habitat but not in others, it was scored as facultative mycorrhizae. The criteria used in this study for the determination of AM was the presence of arbuscules at least in one individual of each species and the occurrence of the rest of typical AM structures in the samples.

Characteristic AM structures (intra or intercellular hyphae, vesicles, coils and arbuscules) were used for their classification into *Arum* or *Paris*-type (Smith and Smith, 1997). The percentage of root length colonized by AM fungi was estimated according to the magnified line-intersect method described by McGonigle *et al.* (1990).

RESULTS

A total of 14 sporophytes of *L. paniculatum*, 12 of them terrestrial and the other two epiphytic, and 12 terrestrial sporophytes of *E. bogotense* were collected. Typical AM fungal structures were observed in six specimens of *L. paniculatum* (43%) and in eight specimens of *E. bogotense* (67%). All colonized sporophytes had knobby hyphae (which were in general coenocyt-
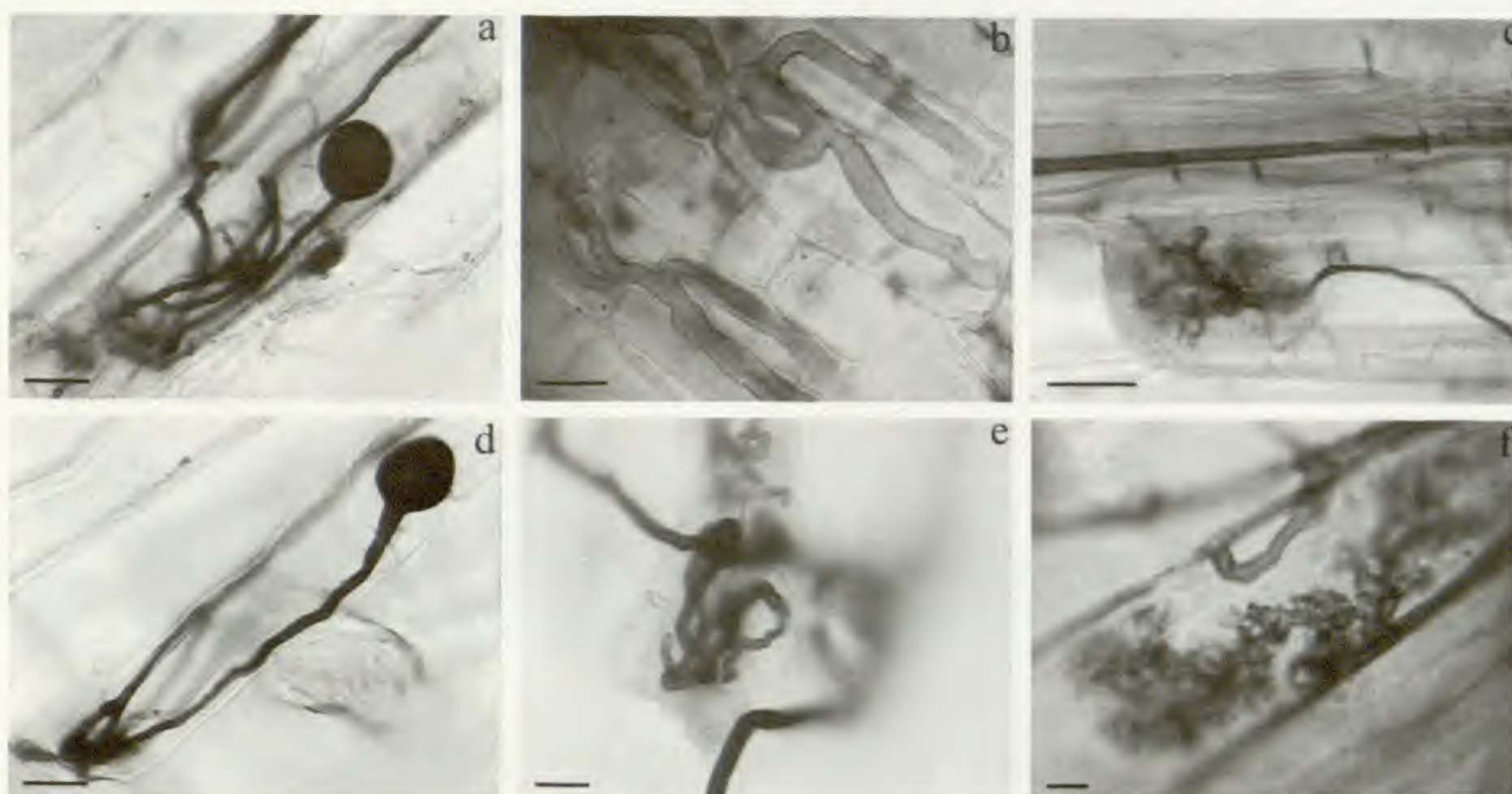
FIG. 1. Arbuscular mycorrhiza in roots of *L. paniculatum* (a-c) and *E. bogotense* (d-f). **a:** Intracellular hyphae and vesicle, **b:** Intracellular coil, **c:** Arbuscule, **d:** Intracellular hyphae and vesicle, **e:** Intracellular coils and cell-to-cell hyphae, **f:** Arbuscule. *Scale bars:* **a**=20 μm; **b**=10 μm; **c**= 5 μm; **d-f**=20 μm.

ic), vesicles (Fig. 1a,d) and some coils characteristic of AM fungi (Fig. 1b,e). Arbuscules were found in four of six (67%) and in six of eight (75%) colonized specimens of *L. paniculatum* (Fig. 1c) and *E. bogotense* (Fig. 1f), respectively (Table 1).

The percentage of root length colonized by AM fungi varied widely within each species. In *L. paniculatum* these values ranged from 0 to 50% with a mean value of 9.5%. Arbuscular mycorrhizas were observed in six of 12 terrestrial sporophytes and epiphytic individuals were not infected. The specimen with the highest value of root length colonized by AM was collected in the forest (50%); however this vegetation unit presented a lower proportion of colonized individuals than the waterlogged peat bog. The specimen that showed the lowest percentage of root length colonized by AM fungi (2%) was found in the waterlogged peat bog (Table 1).

In *E. bogotense* the percentage of root length colonized by AM fungi ranged between 0 and 22.5%, the mean value was 8.6%. All the individuals colonized by AM fungi were collected from the stony edge of the road. Those specimens sampled from sandy soil at the edge of the road and in the coast of the lake were not infected (Table 1).

The AM colonization observed in these two species could not be clearly classified as *Arum* or *Paris*-type. Some intracellular coils were seen in *L. paniculatum* and *E. bogotense* (Fig. 1b,e) and the first species also presented some well-defined, cell-to-cell hyphae. Only one arbuscule within each cell was observed (Fig. 1c, f) and intercalary arbuscules ("arbusculate-coils") were not detected, as would be expected in *Paris*-type pattern. In addition, both species lacked typical *Arum*-type intercellular hyphae. Therefore, the

TABLE 1.   AM colonization in *L. paniculatum* and *E. bogotense*. *References*: **N°**: sample number, **AM (%)**: mean and standard deviation (X ± SD) of the percentage of root length colonized by AM fungi; * positive but non-quantified sample.

Habitat	Place of collection	N°	AM (%)	Arbuscules
Lycopodium paniculatum				
Forest	Soil	1	50.0 ± 27.7	+
		2	0	−
		3	0	−
	Soil (edge of road, stony)	4	26.2 ± 15.8	+
		5	0	−
		6	0	−
		7	0	−
	Trunk	8	0	−
Waterlogged peat bog	Soil	9	22.5 ± 18.6	+
		10	2.0 ± 2.7	−
		11	9.3 ± 8.7	+
		12	0	−
		13	22.9 ± 9.25	−
	Trunk	14	0	−
Equisetum bogotense				
Forest	Soil (edge of road, stony)	1	22.5 ± 5.7	+
		2	12.9 ± 6.1	+
		3	10.2 ± 6.1	+
		4	11.5 ± 5.6	+
		5	+*	−
		6	7.6 ± 4.5	−
		7	12.5 ± 7.7	+
		8	17.1 ± 3.5	+
	Soil (edge of road, sandy)	9	0	−
		10	0	−
	Soil (coast of the lake)	11	0	−
		12	0	−

morphological AM type observed in *L. paniculatum* and *E. bogotense* was considered as an *Intermediate*-type.

Other endophytic fungi were present in all the samples of both species. Most distinctive structures were dark septate intra and extracellular hyphae, microsclerotia and cerebriform structures (Fig. 2), which corresponded to DSF.

DISCUSSION

In this study, it was observed that *L. paniculatum* and *E. bogotense* collected at Puerto Blest were both capable of forming AM. The existence of mycorrhizal as well as non-mycorrhizal individuals within each species supports their facultative behavior, a phenomenon that has been observed in other *Lycopodium* and *Equisetum* species in different regions of the world. For example, *L. japonicum* Thunb. was documented by Zhao (2000) as facultative mycotrophic in China, while Muthukumar and Udaiyan (2000) recorded the
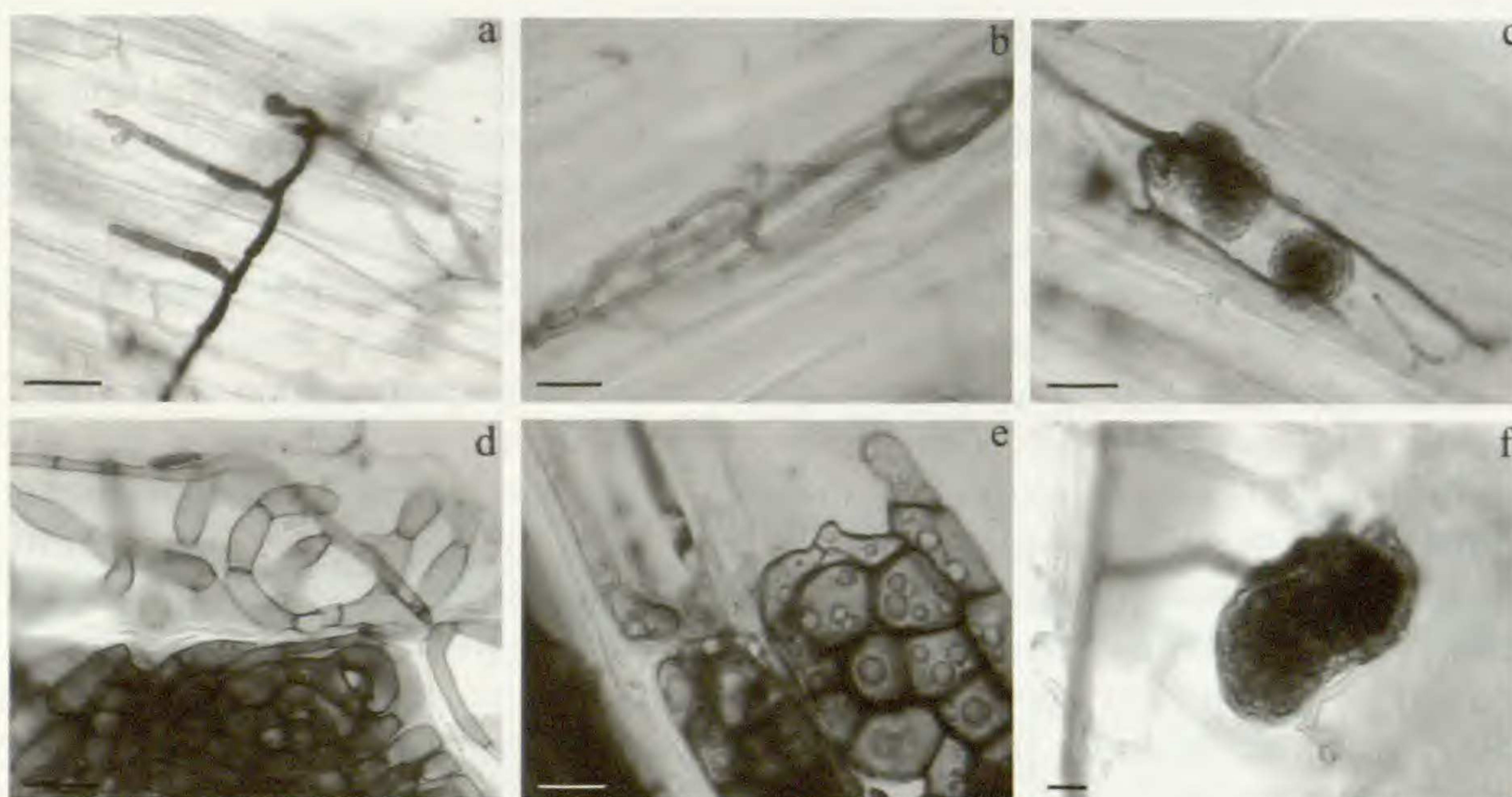
FIG. 2. Dark septate fungi in *L. paniculatum* (a-c) and *E. bogotense* (d-f). **a:** Moniliforms hyphae, **b:** Intracellular microsclerotial structure, **c:** Cerebriform microsclerotia, **d:** Extraradical mycelia, **e:** Intracellular septate hyphae, **f:** Cerebriform microsclerotia. *Scale bars*: **a,b**= 20 μm; c= 5 μm; **d**= 100 μm; **e,f**=20 μm.

same species as non-mycorrhizal in India. In the case of *E. arvense* L., it had AM in Norway and USA (Dhillion, 1993), was described as facultative mycorrhizal in Canada (Berch and Kendrick, 1982) and was considered as non-mycorrhizal in Alaska (Treu *et al.*, 1996). A similar discrepancy has been observed in *L. clavatum* L. and in *E. hyemale* L., *E. scirpoides* Michx. and *E. variegatum* Schleich. (Berch and Kendrick, 1982; Koske *et al.*, 1985; Harley and Harley, 1987; Dhillion, 1993; Treu *et al.*, 1996; Winther and Friedman, 2007). This information is in agreement with Boullard (1957), who carried out one of the most extensive surveys about the mycorrhizal status of seedless vascular plants and established that the mycorrhizal symbiosis within this group ranges from obligate to facultative or non-mycorrhizal. It also accords with the results obtained by Zhao (2000), who studied the mycorrhizal status of 256 species of lycophytes and monilophytes and found out that 16% of them were facultative mycorrhizal.

Although arbuscules are ephemeral structures which may be difficult to see in field-collected roots (Brundrett, 2004), they were found in many sporophytes of both species. The presence of vesicles, hyphal coils and intraradical non-septate hyphae, which are also considered as features of AM colonization by other authors (Duckett and Ligrone, 1992; Dhillion, 1993; Smith and Read, 1997; Zhang *et al.*, 2004), allowed the detection and quantification of AM fungi in samples where arbuscules were not observed.

The colonized specimens of *L. paniculatum* and *E. bogotense* collected in Puerto Blest presented an *Intermediate*-type of AM colonization. This morphological type has been described for other plants (Dickson, 2004), including some other species of lycophytes and monilophytes (Duckett and

Ligrone, 1991; Zhang *et al.*, 2004). Within the Lycophyta, Zhang *et al.* (2004) found an *Intermediate*-type pattern in *Selaginella moellendorfii* Hieron. However, the same authors observed only *Paris*-type AM among the Monilophyta, including two species of *Equisetum* (*E. hyemale* and *E. ramosissimum* Desf.), and Winther and Friedman (2007) cited the same AM morphological type for *L. clavatum*. Therefore, these genera seem to be able to form both *Paris* and *Intermediate*-types of mycorrhizal symbioses.

Although this survey does not allow definitive conclusions about the relationship between AM colonization and habitats or substrates, it was observed that *L. paniculatum* showed a higher number of colonized individuals in the waterlogged peat bog than in the forest. *Equisetum bogotense* had a different symbiotic behavior depending on the type of soil (substrate) where the samples were found; AM were present in specimens growing in stony soil but were absent in those plants collected from moist sandy soil from the edge of the road and from the coast of the lake. A similar trend was previously described by Koske *et al.* (1985) and Dhillion (1993) in different species of *Equisetum*. They suggested that the absence of mycorrhizas in moist sites may be more indicative of the effect of high soil moisture than of the mycotrophic potential of this genus. Therefore, as was observed by Gemma *et al.* (1992) among the seedless vascular plants of Hawaii, the habitat and substrate seem to play an important role in determining the colonization intensity of *L. paniculatum* and *E. bogotense*, but this fact needs to be further studied.

Dark septate fungi were present in every plant of *L. paniculatum* and *E. bogotense* analyzed in this work. These results agree with previous studies that have recorded the occurrence of DSF within the roots of different seedless vascular plant species (Cooper, 1976; Berch and Kendrick, 1982; Dhillion, 1993; Jumpponen and Trappe, 1998), but differ from the results obtained by Winther and Friedman (2007), who did not find any evidence of nonglomalean AM associations in different species included in Lycopodiaceae. The occurrence of cerebriform microsclerotia in *L. paniculatum* and *E. bogotense* is especially remarkable, because these structures have not been previously cited in the literature. As it is becoming important to report and study DSF in order to stress some common features among these fungi and to understand how they influence the host and their relationship with AM fungi, the occurrence of DSF within the roots of these plant species is novel and relevant and supports the idea that some seedless vascular plant are capable of forming plant-fungal associations with a diversity of fungal lineages (Winther and Friedman, 2007). This information would be very useful to elucidate the nature and ecological importance of these poorly known root colonizing fungi (Jumpponen and Trappe, 1998; Jumpponen, 2001; Peterson *et al.*, 2004).

Sporophytes of Equicetaceae and Lycopodiaceae have been regarded as non-mycorrhizal by several authors, however, the results in this study and previous reports indicate that plants of both families may be extensively colonized by AM fungi. *Lycopodium paniculatum* and *E. bogotense* collected in this Valdivian temperate forest of Patagonia are not obligate mycorrhizal species,

but they develop AM under certain conditions, probably related to the habitat and the substrate where they are growing. In spite of the fact that *Paris*-type is the most common type of AM among the seedless vascular plants (Smith and Smith, 1997; Zhang *et al.*, 2004), the sporophytes of *L. paniculatum* and *E. bogotense* were found to be colonized by an *Intermediate*-type of AM. This work represents the first record of AM fungi in lycophytes and monilophytes of the Valdivian rainforest, and constitutes the initial step in the study of the importance of AM fungi and DSF in these groups of plants.

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